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CRY-DASH gene expression is under the control of the circadian clock machinery in tomato

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Abstract Recently a new member of the blue-light photoreceptor family, CRY-DASH, was reported in *Arabidopsis*, though its distinctive biological functions are still unclear. We characterized the *CRY-DASH* gene of tomato and evidenced that its mRNA is expressed in both seeds and adult organs showing diurnal and circadian fluctuations. Moreover, the *CRY-DASH* transcription pattern is altered in both in a *cry1a* mutant and in a transgenic *CRY2* overexpressor suggesting that *CRY-DASH* regulation must be mediated at least partially by an interaction of CRY1a and CRY2 with the timekeeping mechanism.

Keywords: Tomato; Photoreceptors; Cryptochromes; Gene expression; Diurnal rhythms; Circadian rhythms

1. Introduction

The ability of plants to respond to light is achieved through a number of photoreceptor families, which include red and far-red light sensing phytochromes (PHY) and blue-light specific phototropins and cryptochromes (CRY) [1].

Cryptochromes are flavoproteins that share structural similarity to DNA photolyases but lack photolyase activity [2]. Although originally identified in *Arabidopsis*, cryptochromes have now been found in bacteria, plants and animals [3,4]. Most cryptochrome proteins, with the exception of CRY-DASH (or CRY3), are composed of two domains, an amino-terminal photolyase-related (PHR) region and a carboxy-terminal domain (DAS) of varying size [2]. The PHR region appears to bind two chromophores; one chromophore is flavin adenine dinucleotide (FAD) and the other 5,10-methenyltetrahydrofolate (pterin or MTHF) [5,6]. The carboxy-terminal domain of cryptochromes is generally less conserved than the PHR region [2]; CRY-DASH proteins lack the DAS domain [3,7].

In *Arabidopsis*, three cryptochrome genes (*CRY1*, *CRY2* and *CRY-DASH*) have been described so far [7–9]. Plant cryptochromes play an important role in several blue light-regulated developmental processes such as de-etiolation, flowering and flavonoid biosynthesis [10–14]. CRY1 and CRY2 are intimately connected with the circadian clock machinery: *CRY1* and *CRY2* transcript levels are regulated by the clock and the encoded proteins seem to be involved in the input to the clock [15–17].

In tomato (*Solanum lycopersicum*), three cryptochrome genes have been discovered and analyzed in detail so far: two *CRY1*-like (*CRY1a* and *CRY1b*) and one *CRY2* gene [18,19]. The use of transgenic and mutant lines have shed light on the role of tomato cryptochromes in seedling photomorphogenesis, flavonoid and carotenoid accumulation, adult development, fruit pigmentation and flowering [12–14].

The *CRY-DASH* gene, recently characterized in *Arabidopsis* [7], shares little sequence homology with the other cryptochromes and carries an N-terminal sequence which mediates its import into chloroplasts and mitochondria. Furthermore, CRY-DASH lacks the C-terminal domain which is present in most plant cryptochromes. Though its precise physiological function remains to be elucidated, CRY-DASH is likely to function as a further blue light photoreceptor in *Arabidopsis* [7].

In this article, we report the characterization of an *ORF* of tomato which shares high similarity with *Arabidopsis CRY-DASH*. The tomato *CRY-DASH* mRNA is expressed in both seeds and adult organs and undergoes day/night cycles, with peaks of expression at dawn and dusk. Its transcription pattern is altered in a *cry1a* mutant and in a transgenic *CRY2* overexpressor (*CRY2-OX*). In plants transferred for 24 h of continuous light, the *CRY-DASH* transcript still maintains its cycling rhythm, suggesting that it is controlled by the circadian clock machinery.

2. Materials and methods

Solanum lycopersicum (cv *Moneymaker*), *cry1a* and *CRY2-OX* plants [13,14] were grown in a growth chamber for 28 days in long day conditions (LD) (16 h light-25 °C/8 h dark-23 °C). Light intensity of about 100 μmol m^{-2} s^{-1} was provided by Osram (Munich) 11–860 daylight lamps. For continuous light (LL) experiments, plants grown as described above for 28 days were shifted to continuous light at the dawn of 29th day. The aerial parts of three plants for each genotype (wild-type (*Wt*), *cry1a* and *CRY2-OX*) were harvested at the times shown.

Total RNA (1 μg) was retrotranscribed with oligo-dT and Superscript III (Invitrogen), according to the manufacturer's instructions. First strand cDNA (5 ng) was used as template for quantitative real time RT-PCR (qRT-PCR). qRT-PCR assays were carried out with

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gene-specific primers, using an ABI PRISM 7900HT (Applied Biosystems) and the Platinum SYBR Green master mix (Invitrogen), according to manufacturer's instructions. PCR conditions were: 5′ at 95 °C followed by 45 cycles at 95 °C × 15″ and at 58 °C × 60″. Quantification was performed using standard dilution curves for each studied gene fragment and the data were normalized for the quantity of the β-actin transcript.

In situ hybridization was performed on seeds imbibed for 96 h and aerial parts of *Wt* plants grown in LD conditions for 28 days as described above and harvested 12 h after the onset of illumination. Imbibed seeds and tissues (leaves and stems) excised from adult plants were fixed, dehydrated, embedded in paraffin, cut into 8 μm sections and hybridized (55 °C) to a digoxigenin-labelled antisense probe as described by Canas et al. [20]. A gene-specific cDNA fragment of 265 bp was used for the synthesis of the digoxygenin-labelled probe. In parallel, RNA from seeds, leaves, stems and roots was used to monitor *CRY-DASH* transcription by qRT-PCR, following the procedures described above.

3. Results and discussion

3.1. Isolation of the tomato CRY-DASH gene

We have isolated complete genomic and cDNA sequences of a putative new member of tomato cryptochrome gene family, *CRY-DASH* (GenBank Accession No. DQ222242) based on the information available in *Arabidopsis* [7].

Comparison of the genomic and cDNA sequences revealed the presence of a complex gene structure with 13 exons and 12 introns. Most of the exon/intron borders appear to be conserved among angiosperms (Fig. 1).

As already described in *Arabidopsis*, the tomato *CRY-DASH* coding sequence contains a putative targeting sequence for import in organelles (http://www.cbs.dtu.dk/services/ChloroP/; http://urgi.infobiogen.fr/predotar/) (Fig. 2). However, these predictions are not definitive and this aspect deserves further investigation.

Most of the amino acids putatively involved in cofactor interaction are conserved throughout the CRY-DASH subfamily (residues 333, 334, 346, 349, 355, 356, 358, 359, 392, 395, 415 and 466 in Fig. 2). All but one (residue 357) of the amino acids which appear to bind FAD in *Synechocystis* [3] are conserved in all plant CRY-DASH proteins (Fig. 2). Four additional residues (residues 247, 253, 261 and 477 in Fig. 2) which cluster around the FAD binding site in *Synechocystis* are also conserved in all species (Fig. 2).

Despite the high similarity between bacterial class I CPD photolyases and CRY-DASH, especially in the chromophore-binding domain, it is plausible that the actual FAD binding mechanism is different given the fact that two tryptophan residues (residues 393 and 459 in Fig. 2), involved in FAD binding in the *Escherichia coli* photolyase, are replaced with V/L and Y/F residues, respectively, in CRY-DASH proteins [21]. In the same way, FAD binding could also diverge in CRY1–CRY2 like proteins; here most of the residues putatively involved with FAD interaction are, indeed, different with respect to CRY-DASH (data not shown).

Three key tryptophans (residues 427, 480 and 503 in Fig. 2), which probably constitute an electron transfer chain from the photolyase surface to the FAD cofactor [22,23], appear to be highly conserved in CRY-DASH proteins, suggesting that, like in CRY1 and CRY2, their mechanism of action may involve intraprotein electron transfer [24].

Both *Synechocystis* and *Arabidopsis* CRY-DASH [3,7] show a non-specific DNA binding activity. In *Synechocystis* it has been suggested that this activity is mediated by five positively charged arginine residues conserved between CRY-DASH and photolyase (residues 347, 400, 463, 465 and 517 in Fig. 2) [3]. All the above mentioned residues are conserved in the corresponding positions of tomato CRY-DASH, suggesting that a possible DNA binding activity could also occur for the tomato protein. Further experiments are needed in order to prove the specific role of these amino acids in tomato CRY-DASH.

3.2. Tissue-specific gene expression

To determine the histological domains of *CRY-DASH* expression, we performed *in situ* hybridization with digoxigenin-labelled RNA probes. After 96 h of seed imbibition, *CRY-DASH* transcripts were detected both in the endosperm (Fig. 3a) and embryo (Fig. 3a–f). These results are consistent

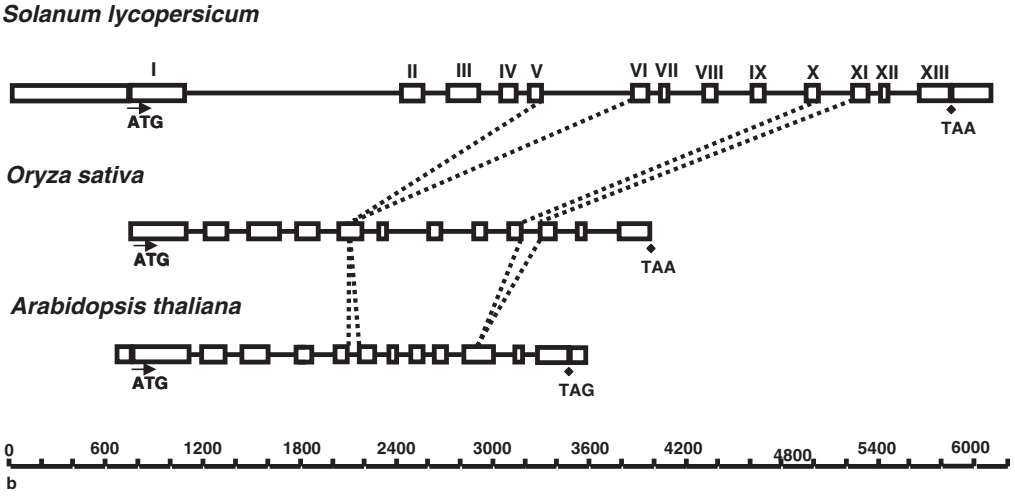

Fig. 1. Comparison of *CRY-DASH* gene structure in *Solanum lycopersicum* (GenBank Accession No. DQ222242), *Arabidopsis thaliana* (GenBank Accession No. AB062926) and *Oryza sativa* (GenBank Accession No. AP004744). Coding regions are boxed and introns are shown as black lines. Non-conserved intron–exon borders are indicated by dotted lines.

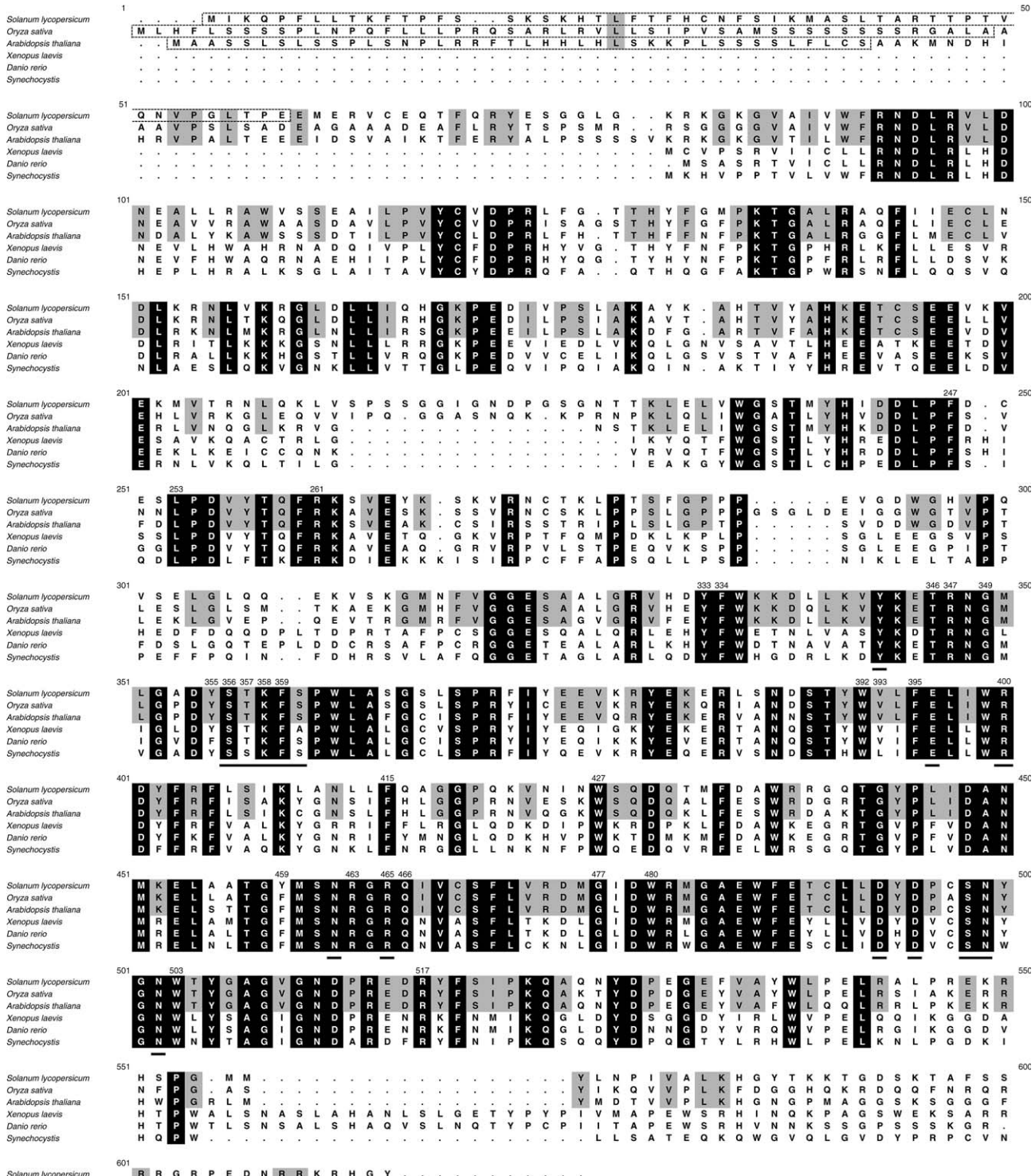

Fig. 2. Multiple alignment of CRY-DASH proteins. Residues conserved across all sequences are shown in inverted type. Residues conserved in plants are shaded in grey. The positions of the amino acids discussed in the text are numbered. Residues involved in FAD binding in *Synechocystis* are underlined. The putative targeting pre-sequences are boxed.

with an early expression of this gene during the resumption of metabolic activity in the germinating seed. Furthermore, in the embryo, transcripts were abundant in the root meristem, along the differentiating vascular strands of the root stele and in the external layers of the root cap (Fig. 3e and f), whereas in the shoot the signal was confined to the tunica layer of Shoot Apical Meristem (SAM) (Fig. 3b). A strong signal was detected on both the abaxial and adaxial epidermis of cotyledons and

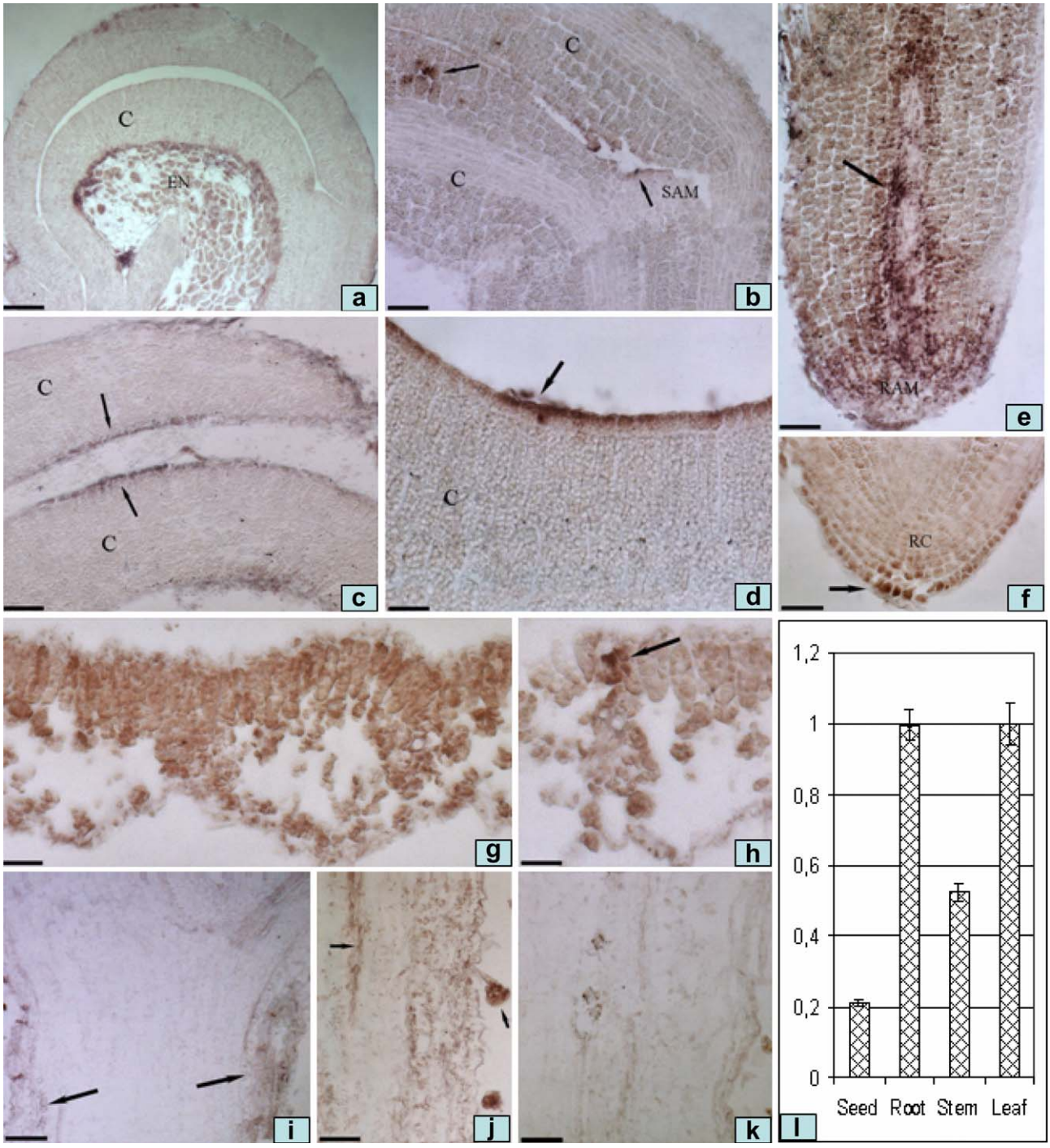

Fig. 3. Tissue-specific transcription of tomato *CRY-DASH* determined by *in situ* hybridization (a–k) and qRT-PCR (l). (a–f) Embryo longitudinal sections; EN = endosperm C = cotyledons; SAM = shoot apical meristem; RAM = root apical meristem; RC = root calyptra; (g, h) leaf cross-sections; (i, j) stem longitudinal sections; (k) control experiment performed with dig-labelled *CRY-DASH* sense probe. Arrows indicate (b) tunica layer in the SAM and cell cluster in the cotyledon, (c, d) epidermis, (e) vascular strand, (f) calyptra external layer, (h) labelled cell cluster, (i) cortex, (j) glandular trichome (on the right) and vascular bundle-associated parenchyma (on the left). (l) qRT-PCR analyses of *CRY-DASH* transcripts in different tissues. Values are normalized for β-actin expression levels and represent means ± S.E. for $n = 3$. All RNA samples were prepared from tissue harvested at ZT12 [30]. Bars: $a = 70$ μm; $b = 22$ μm; $c = 18$ μm; $d = 15$ μm; $e = 27$ μm; $f,j = 21$ μm; $g = 8$ μm; $h = 7$ μm; $i = 16$ μm; $k = 33$ μm.

isolated clusters of expressing cells were observed in the mesophyll tissue (Fig. 3c and d).

In adult plants, a diffuse signal was detected in the whole leaf lamina, at the level of both the palisade and spongy layers (Fig. 3g and h). As for cotyledons, highly expressing clusters of cells scattered along the leaf lamina were often observed (Fig. 3h). In the stem, *CRY-DASH* transcripts were preferentially accumulated in the photosynthetic cortex and at the level of vascular bundle-associated parenchyma cells (Fig. 3i and j). *CRY-DASH* transcripts were highly abundant in glandular trichomes (Fig. 3j). Finally, no transcripts were detected with the *CRY-DASH* sense RNA probe (Fig. 3k).

In parallel, using the same plants, we monitored *CRY-DASH* transcript levels in root, stem, leaf and seed tissues by qRT-PCR. Although *CRY-DASH* mRNAs were detectable in all samples, transcripts were about fivefold and twofold higher in root and leaf, with respect to seed and stem tissues, respectively (Fig. 3l).

In principle, the wide range of tissues and organs in which *CRY-DASH* resulted transcriptionally active is consistent with a multiple biological role of CRY-DASH either as a possible further blue-light photoreceptor and/or as an element involved in the regulation of diurnal and circadian rhythms.

3.3. Day/night and circadian transcription fluctuation of CRY-DASH transcripts

In silico analysis of the tomato *CRY-DASH* promoter predicts the presence of several light-regulated transcription factor binding sites. Among these, GT-1 and GATA motifs have been shown to be very important in light-regulation of gene expression [25–27] (Fig. 4). The tomato *CRY-DASH* promoter also contains two CCA1 putative binding sites, both containing the AATCT core motif [27]. One of these (AAAATCT) is a morning-phased promoter site [28] (Fig. 4). It is remarkable that just upstream of these motifs is present a light-induced-circadian G-box GCCACGTGTC [26]. Typically, all these motifs are part of light/circadian-regulated gene promoters and usually cooperate in defining the transcript oscillation properties [25,26,28] (Fig. 4).

The presence of a morning-phased CCA1 binding site is not in contrast with the biphasic *CRY-DASH* expression pattern, showing peaks at dawn and at dusk (see below). It should be considered that the actual transcript oscillation pattern is usually the result of the concomitant positive and negative action of different cis-acting elements [29].

In order to characterize possible patterns of light regulation and rhythmic transcript oscillations, we measured, via qRT-PCR, *CRY-DASH* mRNA levels at 4-h intervals in plants grown in a diurnal cycle of 16 h light/8 h dark (LD) (Fig. 5a). Plants that had been grown in an LD (16:8) cycle were put at dawn in continuous light (ZT0) and left there for 40 h measuring circadian transcript oscillation at 4-h intervals (Fig. 5b). The mRNA levels were measured in the *Wt*, in a *cry1a* mutant [13] and in a transgenic *CRY2* overexpressor (*CRY2-OX*) [14]. Sampling time is expressed as *Zeitgeber time* (ZT) [30], which is the number of hours after dawn (the onset of illumination).

In the *Wt*, *CRY-DASH* transcript levels oscillate under LD conditions. They are relatively high at ZT0, decrease from ZT0 to ZT4, then increase again from ZT4 to the afternoon (ZT12) and then progressively decrease from ZT12 to ZT20. Overall, they show two peaks (at ZT0 and 12) and two troughs (at ZT4 and 20) (Fig. 5a). In *cry1a* and *CRY2-OX* plants, the peak at ZT0 is abolished, and the one at ZT12 is reduced in amplitude. *Cry2-OX* plants also show a delayed, lower amplitude dawn-phased peak (Fig. 5a).

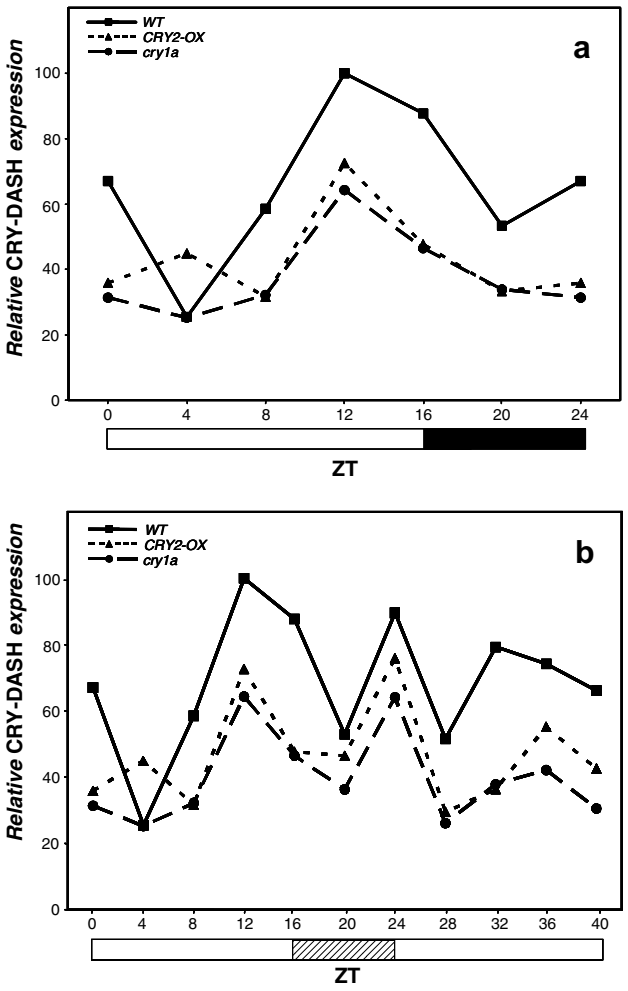

Fig. 5. Transcription analysis of tomato *CRY-DASH* gene analyzed by qRT-PCR in *Wt*, *cry1a* and *CRY2-OX* plants grown in LD (a) and LL (b) conditions. Results are presented as a proportion of the highest value after normalization with respect to actin expression levels. Open, closed and hatched bars along the horizontal axis represent light, dark and subjective night periods, respectively; these are measured in hours from dawn (zeitgeber Time – ZT) [30]. Each experiment was done at least twice with similar results.

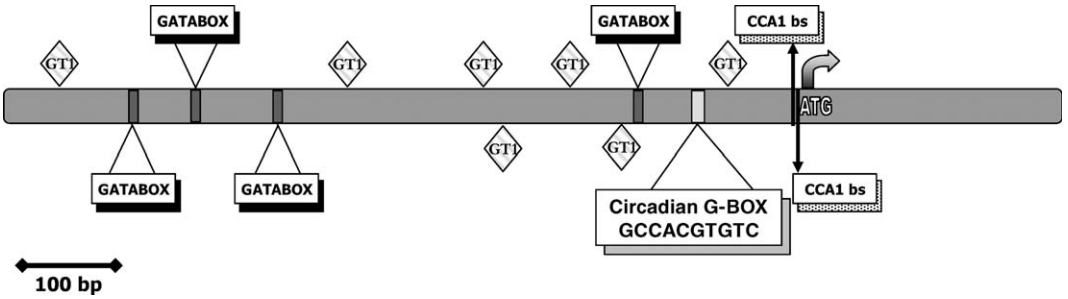

Fig. 4. Graphical representation of the DNA region upstream the putative start codon of the tomato *CRY-DASH* gene. A number of possible targets for light-responsive elements have been identified and reported within boxes (see references in the text).

Under continuous light (LL) conditions, some similarities and some differences are observed with respect to those grown in LD conditions:

- The oscillations observed in LD (decrease from ZT16 to ZT20, subsequent increase until ZT24 and decrease until ZT28) are still observed (Fig. 5b), indicating that they controlled by an endogenous clock.
- The expected peak at ZT36 is anticipated by 4 h (ZT32) in LL conditions (Fig. 5b), indicating that light has a partial resetting effect on the transcription rhythm.
- In *cry1a* and *CRY2-OX* plants, the peak at subjective dawn is observed in LL (ZT24), but not LD conditions (ZT0) (Fig. 5a and b), indicating that external light is able to restore a circadian signal present in the *Wt* but absent in these two genotypes.
- Intriguingly, the effect of the *cry1a* and *CRY2-OX* genetic backgrounds on *CRY-DASH* transcription is similar.

These observations allow the following conclusions:

(i) *CRY-DASH* gene transcription responds, directly or indirectly (e.g. through the clock machinery), to environmental light and to endogenous circadian signals.

(ii) CRY1a and CRY2 mediate part of these responses, albeit in antagonistic ways. In LD conditions, CRY1a stimulates and CRY2 represses gene transcription during the whole cycle, particularly between ZT20 and ZT24. Since no light is present at this time, these responses must be mediated at least partially by an interaction of CRY1a and CRY2 with the timekeeping mechanism.

(iii) Under LL, but not LD conditions, *cry1a* and *CRY2-OX* plants show an induction similar to the *Wt* between ZT20 and ZT24, suggesting that this induction is presumably mediated by a photoreceptor different from CRY1a and CRY2. Under LL, this photoreceptor is substituting for the clock function that normally produces this peak in *Wt* seedings.

Many authors suggest a specific role for cryptochromes 1 and 2 in light input to the circadian clock; however, the molecular mechanism that transmits light signals to the clock is not yet clear [16]. The dawn–dusk phased expression pattern of tomato CRY-DASH, especially in *Wt* under LL conditions is very intriguing (Fig. 5b). In fact, this pattern is consistent with a potential role for CRY-DASH in detecting the dawn and dusk transitions and, consequently, in circadian input pathways.

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